REMARKS

I. Preliminary Remarks

Claims 1, 3, 4, 6-11 and 16-19 are pending with claims 6-11 being withdrawn from consideration for being drawn to a non-elected invention. Claims 1, 3, 4 and 16-19 are under examination.

II. Interview Summary

Applicants thank Examiners Emch and Kemmerer for the telephone interview with the undersigned and David A Gass on October 4, 2007. During the interview, the Powell reference was discussed and agreement was reached. Examiners Emch and Kemmerer agreed with the Applicants that Powell does not anticipate any of claims 1, 3, 4, and 16-19.

III. The rejection under 35 U.S.C. § 112, first paragraph, is moot and should be withdrawn.

The Examiner maintained the rejection of claims 16, 18 and 19 as allegedly being drawn to subject matter not supported by the specification as filed. It appears that the Examiner has withdrawn the rejection with respect to claims 1, 3, 4 and 17.

At the outset, the Applicants dispute the PTO's assessment that claims 16 and 17 have only one structural limitation, the definition of position 130. Claim 17 required identity across the protein sequence's length to SEQ ID NO: 4 and claim 16 required satisfaction of stringent hybridization conditions. Also, the requirement of aspartyl protease activity imposes a structural constraint on, e.g., the minimum size of the protein.

While Applicants continue to disagree with the Examiner regarding the support found in the application for these claims, claims 16, 18 and 19 have been amended to expedite prosecution of this application. In particular, claim 16 has been amended to recite specific hybridization conditions and claims 16, 18 and 19 have been amended to include additional structural language and now recite "and comprises aspartyl protease active site tripeptides DTG and DSG." Support for amended claims 16, 18 and 19 can be found throughout the specification and in claim 1.

There is only one DTG and one DSG in SEQ ID NO: 4, so the genus defined in claims 16, 18 and 19 is well defined by SEQ ID NO: 4. Fragments that include the aspartyl protease active site tripeptides DTG and DSG are described in the specification at page 30, lines 9-19. The application also provides aspartyl protease assays and substrates for the enzyme. Thus, the

specification adequately describes the biologically-active aspartyl proteases recited in claims 16, 18 and 19.

The limitation of claim 16 specifying that the polypeptide is encoded by nucleic acid molecule that hybridizes under the specified stringent hybridization conditions to the complement of SEQ ID NO: 3 defines a reasonable number of variants encompassed by the genus of claim 16. Example 9 of the Patent Office's Written Description Guidelines Training Materials provides a hypothetical invention involving nucleic acid sequence that hybridize under highly stringent conditions to the complement of a disclosed sequence and the nucleic acid encodes a protein with a recited activity. The Patent Office's opinion regarding such a claim is that one of skill in the art would not expect substantial variation among the species encompassed by the claim because the recited hybridization conditions yield structurally similar nucleic acids. Therefore, the disclosed sequences are considered a representative number of species in view of the structural and functional requirements of the claim and therefore the genus of polypeptides is adequately described. The Patent Office's analysis of the written description requirement in the hypothetical does not concern itself with whether the specification provided specific guidance about specific changes at specific positions in the sequence because the genus is sufficiently described by the hybridization limitation, the reference sequence and the biological activity. Or stated differently, the limitations used by the Applicants in claim 16 have been recognized by the Patent Office to provide sufficient specificity and limit variability enough to satisfy the written description requirement.

In view of the foregoing, the rejection of claims 16, 18 and 19 under 35 U.S.C. § 112, first paragraph, is moot and should be withdrawn.

IV. The rejection under 35 U.S.C. § 102(e) should be withdrawn.

The Examiner maintained the rejection of claims 1, 3, 4, and 19 under 35 U.S.C. § 102(e) as assertedly being anticipated by Powell (U.S. Patent No. 6,319,689) as evinced by Vassar (Adv. Drug Delivery Rev., 54:1589-1602, 2002).

As discussed in the telephonic interview with Examiners Emch and Kemmerer on October 4, 2007, the rejection of claims 1, 3, 4, and 19 should be withdrawn because Powell does not expressly or inherently disclose the polypeptides recited in the claims. "A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." Verdegaal Bros. v. Union Oil Co. of Cal., 2 U.S.P.Q.2d 1051, 1053 (Fed. Cir. 1987); see also M.P.E.P. § 2131.

Powell only discloses one sequence (i.e., the full-length amino acid sequence of SEQ ID NO: 2) for an alleged aspartyl protease, and this sequence does not anticipate any claim. For example, claim 1 is drawn to a purified or isolated polypeptide comprising (a) the amino acid sequence set forth in SEQ ID NO: 4; (b)fragments of (a) that exhibit aspartyl protease activity in processing APP into amyloid beta and includes aspartyl protease active site tripeptides DTG and DSG; or (c) conservative substitution variants of (a) or (b); and requires that amino acid substitutions (if any) in the claimed polypeptides, relative to the fragment of SEQ ID NO: 4, be conservative substitutions. The substitution difference between Powell's sequence and SEQ ID NO: 4 is not a conservative substitution: Powell's sequence has a Glu at position 130, while SEQ ID NO: 4 has a Val at position 130. A substitution of Val for Glu is not a conservative substitution, as Glu is an acidic residue and Val is an aliphatic residue. Accordingly, the genus of substitution variants of SEQ ID NO: 4 encompassed by the claims do not include Powell's sequence, and Powell cannot anticipate claim 1 or any claim dependent thereon.

Powell contains more than a sequence, of course, but the Examiner has failed to identify any specific teachings in Powell that anticipate any of the claims. In particular, the Examiner has failed to identify specific teachings in Powell of: (1) any active fragment of Powell's Asp2; (2) an active fragment in which position 130 of its sequence is deleted; or (3) the recognition of Powell of the existence of an Asp2 transmembrane domain, or a specific fragment characterized by the removal of the transmembrane domain.

The Examiner asserts that Powell teaches the active site tripeptides and fragments of its Asp2 polypeptide that retain aspartyl protease activity. Applicants disagree. While Powell allegedly contemplates or suggests (in general terms) variants and fragments of its full length Asp2 amino acid sequence, Powell provides no disclosure of any specific fragments and certainly does not provide any disclosure of any specific fragments having aspartyl protease activity as asserted by the Examiner. Mere suggestion of "active" variants/fragments cannot anticipate the specific genera of polypeptides recited in the claims. In fact, Powell is non-enabling with respect to "active" fragments or variants because Powell fails to disclose or suggest any specific activity of its Asp2 polypeptide. Powell generically defines the activity of its Asp2 polypeptide as having "the metabolic or physiologic function of said Asp2, including similar activities or improved activities or these activities with decreased undesirable side-effects. Also included are antigenic and immunogenic activities of said Asp2." (See Powell, col. 1, lines 59-64). Powell "expects" Asp2 polypeptides to have "similar biological functions/properties" to homologous peptides and makes specific reference to an allegedly novel Asp1 polypeptide, which itself has unreported function and less than 50% amino acid identity to

Asp2. (See cols. 7-8 of Powell.) Powell makes no mention of its Asp2 having aspartyl protease activity at all and is silent with respect to Asp2's involvement in processing APP into amyloid beta (or processing any other substrate). The present application is the first disclosure of such activities. Consequently, the Examiner cannot rely on Powell for providing an explicit or inherent disclosure of the active polypeptides recited in the claims. Powell is arguably non-enabling for any activity, and is unquestionably non-enabling with respect to fragments having the APP processing activity required by the current claims.

The Examiner further asserts that "a polypeptide that includes a fragment of the Asp2 polypeptide of the Powell et al. reference ***could*** contain the active site tripeptides without residue 130, thus meeting the limitations of claim 1. (emphasis added)" See Office Action, page 10. First, such speculation is irrelevant. The Examiner has not set forth a *prima facie* case of anticipation because the Examiner has not identified a polypeptide taught in Powell that allegedly meets all of the limitations of the claims and lacks Powell's Glu130 residue! Speculation about what a person "could" make has no relevance to whether an allegedly anticipatory reference actually discloses an invention, as required to anticipate a claim. Powell does not teach such a polypeptide.

The Examiner relies upon the instant specification to provide disclosure not provided by the cited art. In particular, the Examiner asserts "[a]ccording to Applicants' own specification, functional fragments that contain the residues between the active sites may be shortened or lengthened, as long as the active sites are maintained." Even if the Examiner were permitted to rely on teachings of the pending application to interpret the prior art (a proposition that the applicants dispute), the present application does not teach that position 130 may be deleted. Even if the current application did teach this, it would not render Powell a novelty reference. The Powell reference must be the source of the alleged novelty-destroying teachings, and the Powell reference does not teach a fragment that has active site tripeptides and lacks Glu130.

As summarized in MPEP 2112, "the fact that a certain result or characteristic may occur or be present in the prior art is not sufficient to establish the inherency of that result or characteristic." (Emphasis in original); In re Rijckaert, 9 F.3d 1531, 1534, 28 USPQ2d 1955, 1957 (Fed. Cir. 1993), In re Oelrich, 666 F.2d 578, 581-582, 212 USPQ 323, 326 CCPA 1981). Besides the mere recitation of DTG and DSG in Powell's sequence, Powell does not disclose or suggest that DTG and DSG are required for aspartyl protease activity involved in the processing of APP into amyloid beta and certainly does not disclose or suggest that a fragment containing residues between DTG and DSG can be shortened and still remain active, as asserted by the Examiner. Powell provides

no disclosure to suggest that any fragment would have the activity recited in the claims much less a fragment lacking residue 130, and therefore does not support the Examiner's assertion that such fragments could be active. The Examiner is reminded that "inherency . . . may not be established by probabilities or possibilities." *See Purdue Pharma v. Boehringer Ingleheim 98 F.Supp. 2d 362, 379 (SDNY, 2000), citing Mehl/Biophile Int'l Corp., 192 F.3d at 1365).*

The Examiner relies on Vassar to establish that the activity recited in the claims (i.e., processing APP into amyloid beta) is an inherent property of Powell's Asp2 sequence. This is factually incorrect because Vassar does not purport to teach anything about Powell's Asp2 sequence. Figure 2 of Vassar discloses the amino acid sequence of SEQ ID NO: 4 of the present invention and not the amino acid sequence taught in Powell. Accordingly, because Vassar does not disclose or suggest Powell's sequence, Vassar cannot provide inherent properties for Powell's sequence.

The Examiner further asserts that "Powell teaches soluble fragments of the Asp2 polypeptide (Col. 20, line 25), which are polypeptides that lack the transmembrane domain. Thus, it is irrelevant that the Powell reference does not explicitly recite removing a transmembrane domain." Powell fails to disclose or suggest that its Asp2 has a transmembrane domain at all, fails to disclose or suggest whether its Asp2 is soluble or insoluble, and fails to disclose or suggest what portions of its Asp2 to remove to make it soluble. Importantly for the purposes of anticipation analysis, Powell fails to disclose any specific fragment that lacks the Asp2 transmembrane domain.

Powell also fails to disclose, suggest, or enable removal of the transmembrane domain. Based on previously described aspartyl proteases, including those cited in Powell, one of ordinary skill in the art would not have expected the aspartyl protease to contain a transmembrane domain. However, the failure of Powell to teach a transmembrane domain does not mean that the Powell polypeptide does not have a transmembrane domain. The polypeptide disclosed in Powell does, in fact, have a transmembrane domain. This domain is located at approximately positions 454-480 of Powell's Asp2 amino acid sequence. In a subsequent publication authored by Powell, the Asp2 sequence is characterized as "a transmembrane aspartic protease." Charlwood et al., J. Biol. Chem., 276:16739-48, 2001 (set forth in Appendix A).

One consequence of Powell's failure to teach a transmembrane domain in its polypeptide is that Powell certainly does not teach to remove a transmembrane domain for any reason. Further, Powell does not disclose any specific Asp2 fragment that lacks the region identified by the Applicants as a transmembrane domain of Asp2, yet retains the active site tripeptides and exhibits aspartyl protease activity, as claimed in the present application. Thus, an Asp2 polypeptide that lacks

a transmembrane domain, yet retains the active site tripeptides and retains aspartyl protease activity, is novel over Powell.

Finally, Powell does not provide an enabling disclosure because it does not demonstrate a biological activity of the Asp2 polypeptide. Importantly, Powell fails to disclose or suggest substrates for the putative enzyme. Thus, without the disclosure of an APP substrate, Powell cannot be construed as disclosing or suggesting fragments of Asp2 that are active against APP. Similarly, because Powell fails to disclose or suggest that its Asp2 sequence has a transmembrane domain, Powell cannot be construed as disclosing or suggesting active Asp2 fragments lacking the transmembrane domain. Because of Powell's failure to characterize the biological activity or other properties of its Asp2 polypeptide, failure to identify a role for the enzyme in APP processing, failure to disclose the presence and removal of a transmembrane domain and other shortcomings, Powell does not provide an enabling disclosure for the subject matter upon which the Examiner has relied.

For all of these reasons, Powell does not anticipate any of the pending claims and the rejection should be withdrawn.

V. The obviousness-type double patent rejection is moot.

Claims 1, 3, 4 and 16-19 were rejected under the judicially created doctrine of obviousness-type double patenting in view of the following patents: U.S. Patent Nos. 6,913,918, 6,825,023 and 6,828,117. In addition, claims 1, 3, 4 and 16-19 were provisionally rejected under the judicially created doctrine of obviousness-type double patenting in view of the co-pending patent application nos. 10/652,830 and 10/940,867. The rejections are moot in view of the terminal disclaimers submitted herewith.

VI. Conclusion

Please charge Deposit Account No. 13-2855, under order no. 29915/6280N3 in the amount of $1,460.00 covering the fees for the filing of five terminal disclaimers ($650.00) and the fee associated with a request for continued examination ($810.00). The Director is authorized to charge any additional fees deemed necessary to Deposit Account No. 13-2855, under order number 29915/6280N3.

If the Examiner believes that a telephone conversation would expedite allowance of the claims, she is invited to contact the undersigned agent or David A. Gass, Attorney for Applicants, at the number below.

Dated: October 23, 2007

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APPENDIX A

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Characterization of the Glycosylation Profiles of Alzheimer's β-Secretase Protein Asp-2 Expressed in a Variety of Cell Lines*

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Amyloid 39–42 β-peptides are the main components of amyloid plaques found in the brain of Alzheimer's disease patients. Amyloid 39–42 β-peptide is formed from amyloid precursor protein by the sequential action of β- and γ-secretases. Asp-2 is a transmembrane aspartic protease expressed in the brain, shown to have β-secretase activity. Mature Asp-2 has four *N*-glycosylation sites. In this report we have characterized the carbohydrate structures in this glycoprotein expressed in three different cell lines, namely Chinese hamster ovary, CV-1 origin of SV40, and baculovirus-infected SF9 cells. Biantennary and triantennary oligosaccharides of the "complex" type were released from glycoprotein expressed in the mammalian cells, whereas mannose-rich glycans were identified from glycoprotein synthesized in the baculovirus-infected cells. Site-directed mutagenesis of the asparagine residues at amino acid positions 153, 172, 223, and 354 demonstrate that the protease activity of Asp-2 is dependent on its glycosylation.

One of the key pathological features of Alzheimer's disease is the formation of brain plaques primarily due to the fibrilization of amyloid 39–42 β-peptides (Aβ)[1] (1, 2). The formation of these peptides by the action of proteases on amyloid precursor protein (APP) has been the subject of several studies (3, 4), as the identification of these proteases could lead to the discovery of inhibitors useful in the treatment of Alzheimer's disease. Over the last year there have been a number of reports detailing the discovery, purification, and characterization of a transmembrane aspartic proteinase (Asp-2). This glycoprotein has been shown to cleave the APP at the β-secretase site (5–9). High levels of this proteinase were identified in the human brain, and the enzyme activity was found in cells associated with the central nervous system (7) and in cell lines known to produce Aβ via cleavage of APP (5).

Asp-2 contains four potential *N*-linked glycosylation sites at the following asparagine residues: Asn^{153}, Asn^{172}, Asn^{223}, and Asn^{354}. The close proximity of some of these glycosylated sites to the three intramolecular disulfide linkages and the catalytic site of Asp-2 has been the subject of more recent interest (10), especially because the type and extent glycosylation of a protein can have a profound effect on its physico-chemical properties (11). For instance, it is well known that *N*-linked oligosaccharides can influence glycoprotein folding in the endoplasmic reticulum (12) and can protect a protein from protease attack (13).

The *N*-linked oligosaccharide structures on Asp-2 have not yet been characterized in detail, although it has been reported that all four asparagine sites have a heterogeneous mixture of carbohydrate attached (10). Another report has indicated that the glycans in mature Asp-2 are of the complex type, as removal of this carbohydrate is not possible by endoglycosidase H treatment (14).

In this study we have released and analyzed the *N*-linked glycans from Asp-2 expressed as an Fc fusion in CHO and COS cells, expressed in baculovirus-infected insect cells and expressed in CHO cells after subsequent proteolytic removal of the Fc moiety. We have identified the different oligosaccharides attached to Asp-2 and demonstrate that the heterogeneity in glycan distribution is very similar when Asp-2 is expressed in mammalian cell systems; under these conditions, only complex-type glycans are attached to the asparagine residues of the four glycosylation motifs found in the primary sequence of this protease. We have also used site-directed mutagenesis to ascertain that glycosylation makes a significant contribution to the activity of Asp-2.

EXPERIMENTAL PROCEDURES

Materials—Gradient Tris-glycine (4–20%) slab gels were purchased from NOVEX (San Diego, CA). PNGase F was obtained from Roche Molecular Biochemicals (Lewes, United Kingdom (UK)). Dithiothreitol was obtained from Calbiochem (La Jolla, CA). *Arthrobacter ureafaciens* sialidase was obtained from Glyko (Novata, CA). The following samples of Asp-2 (numbering from the initiator methionine) and the corresponding Fc fusion proteins were used for glycan release: Asp-(1–501), wild-

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[1] The abbreviations used are: Aβ, amyloid β-peptide(s); AA-Ac, 3-(acetylamino)-6-aminoacridine; APP, amyloid precursor protein; Asp-2, transmembrane aspartic protease; CHO, Chinese hamster ovary; COS, CV-1 origin of SV40; HILC, hydrophilic interaction liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; $Man_{3-9}GlcNAc_2$, neutral glycans containing 3–9 mannose and 2 *N*-acetylglucosamine residues; PNGase F, peptide *N*-glycanase; MS, mass spectrometry.

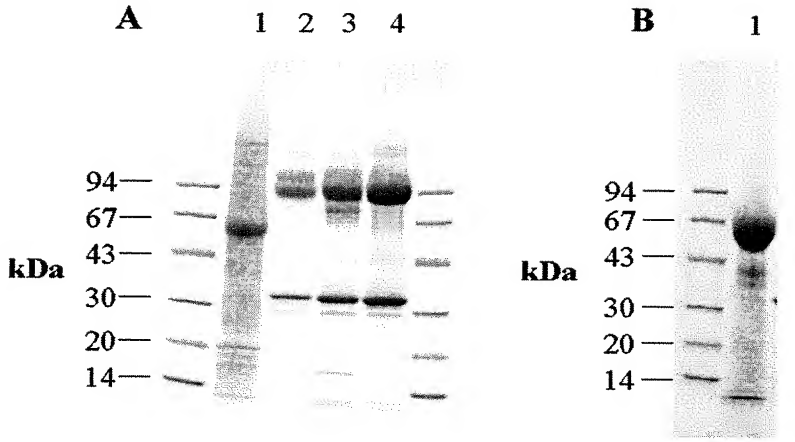


FIG. 1. **Recombinant Asp-2 produced in different expression systems.** The positions of the marker proteins and their estimated molecular masses are indicated on the *left*. *A*, *lane 1*, wild-type Asp-2-His_6 expressed in baculovirus-infected SF9 cells; *lane 2*, Asp-2Fc expressed in COS cells; *lanes 3* and *4*, Asp2Fc expressed in CHO E1a cells. *B*, *lane 1*, purified Asp-2Fc in CHO E1a cells and proteolyzed to remove the Fc. The protein bands at ~38 and 40 kDa have been generated by minor COOH-terminal degradation of the recombinant protein in both mature and proprotein forms.

type full length Asp-2 expressed in baculovirus-infected SF9 cells; Asp-(1–460Fc), Asp-2Fc expressed in COS and CHOE1a cells; Asp-(1–460), Asp-2Fc, expressed in CHO E1a cells, purified and proteolytically processed to remove the Fc.

Expression and Purification of Asp-2 in Mammalian Cells—The various Asp2 Fc expression constructs were transiently transfected into COS-1 cells using LipofectAMINE Plus (Life Technologies, Inc.) according to the manufacturer's instructions with minor modifications. For stable cell line selection, the plasmids were linearized by digestion with *Not*I (15 μg of DNA, 37 °C, overnight), precipitated and resuspended into 50 μl of 1× TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5). The DNA was electroporated, using a Bio-Rad Gene Pulser into a CHO E1A cell line (derived from DG-44 (15) adapted for growth in suspension in maintenance medium) using the technique of Hensley *et al.* (16). The cells were plated into 96-well culture plates at 5×10^5 cells/plate in maintenance medium for 24 h prior to selection. Cells were selected in maintenance medium without nucleosides (selection medium). Conditioned medium from individual colonies was assayed using an electrochemiluminescence detection method on an Origen analyzer (IGEN) the technology reviewed by Yang *et al.* (17). A colony expressing high levels of Asp-2 was used to inoculate 100 ml of selection medium to generate conditioned medium from which Asp-2 Fc was purified by affinity chromatography on immobilized Protein A.

Expression and Purification of Asp-2 in Baculovirus-infected SF9 Cells—The cDNA encoding Asp2 (amino acids 1–501) with a COOH-terminal six-histidine tag was cloned into pFastbac (Life Technologies, Inc.). Recombinant virus was generated according to manufacturer's protocols. Protein was purified from SF9 cells (Life Technologies, Inc.), which were infected with virus as described, and Asp2 protein was purified by metal chelating and lentil lectin chromatography.[2]

Generation of Expression Constructs and Site-directed Mutagenesis—The cDNA encoding amino acids 1–460 of Asp-2 (GenBank℠ accession no. AF204943) was subcloned upstream of the cDNA encoding amino acids 99–330 of human IgG1, and this construct was subcloned into the mammalian expression vector pCDN (18). To generate the appropriate glycosylation mutants (see Table I for mutations), the Quikchange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used according to manufacturer's instructions. All mutations were confirmed by sequence analysis, and the mutated cDNA was recloned into the parental vector.

Activity Analysis—Peptide (Swedish variant ISEVNLDAEFRHD-K(dnp)G) (50 μM) was incubated with Asp2-Fc and Asp2-Fc mutants (50 nM) in buffer containing 50 mM sodium acetate, 20 mM NaCl, pH 4.5, for 30 min in a final assay volume of 100 μl. The reaction was stopped by addition of four volumes of 5% trifluoroacetic acid. The assay components were loaded onto a POROS R1 column (Applied Biosystems) in 0.08% trifluoroacetic acid and eluted with a linear gradient of 0.08% trifluoroacetic acid in acetonitrile (linear gradient from 5% to 50% acetonitrile over 15 min). The chromogenic dnp group on the peptide and product was followed by monitoring at 360 nm. Enzyme concentrations were determined by absorbance at 280 nm in each case.

Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Gel Electrophoresis—Proteins (10–20 μg) were separated on one-dimensional SDS-polyacrylamide gels using 4–20% gradient Tris-glycine polyacrylamide slab gels (19) (10 cm × 8 cm × 1.0 mm; Novex, San Diego, CA). Proteins in the gels were stained with Coomassie Brilliant Blue R250 dye.

Protein Reduction and Alkylation—The Coomassie-stained bands corresponding to the proteins of interest were excised from the gel and washed for 30 min in distilled water. The gel slice was dehydrated in acetonitrile for 10 min. The acetonitrile was removed and the gel slice dried for 10 min in a vacuum centrifuge. The dried gel slice was incubated first in 20 mM Tris-HCl, pH 8.0, containing 10 mM dithiothreitol for 1 h at 55 °C, and second with 20 mM Tris-HCl, pH 8.0, containing 55 mM iodoacetamide for 45 min in the dark at room temperature. The supernatant was removed and the gel slice washed in 20 mM Tris-HCl, pH 8.0, for 10 min. After removal of the solution, the gel was dehydrated in acetonitrile and dried as before.

Enzymatic Release of Glycans—The reduced and alkylated gel slice was then re-hydrated for 30 min in 50 mM sodium phosphate buffer, pH 7.2, and dehydrated again with acetonitrile. The gel piece was then incubated with 40 μl of 50 mM sodium phosphate buffer, pH 7.2, containing 1 unit of PNGase F (Roche Molecular Biochemicals) overnight at 37 °C.

Extraction of Glycans after in Situ Digestion—Glycans were extracted from gel slices in 5% v/v formic acid for 1 h, followed by three extractions with 60% v/v acetonitrile in 5% v/v formic acid for 1 h (with intermittent sonication). The combined extracts were dried in a vacuum centrifuge.

Derivatization of Glycans with 3-(Acetylamino)-6-aminoacridine (AA-Ac)—Extracted glycans were labeled with AA-Ac by incubating them with 10 μl of an AA-Ac solution (2.5 mg in 200 μl of dimethyl sulfoxide/acetic acid (17:3)) and 10 μl of sodium cyanoborohydride (12.5 mg in 200 μl of dimethyl sulfoxide/acetic acid (17:3)) for 30 min at 80 °C. The reaction was stopped by drying in a vacuum centrifuge. Excess reagent was removed on an OASIS cartridge (Waters). The column was primed with 2 ml of acetonitrile followed by 2 ml of water. The sample was loaded in 200 μl of water and washed with 1 ml of water. The derivatized glycans were eluted with 2× 600 μl of 20% v/v acetonitrile/water and dried in a vacuum centrifuge.

Digestion of Glycan Pools with Sialidase—AA-Ac-labeled oligosaccharides were digested with sialidase by incubating them with 20 μl of *Arthrobacter ureafaciens* sialidase (0.2 units in 100 μl of 100 mM sodium acetate, pH 5) for 18 h at 37 °C. The resulting derivatives were freeze-dried and desalted using an OASIS cartridge.

Hydrophilic Interaction Liquid Chromatography (HILC)—HILC was carried out on a Waters Alliance 2690 Separations module using a GlycoSep N column (Oxford GlycoSciences, 25 cm × 3.9 mm, inner diameter). The mobile phases were acetonitrile (solvent A) and 250 mM ammonium formate, pH 4.4 (solvent B).

The following gradient elution conditions were used: step 1, 65% A, 35% B (equilibration), 0.4 ml/min; step 2, 35–39% B for 50 min, linear gradient; step 3, 39–58% B for 30 min, linear gradient; step 4, 58% B to 100% B for 3 min; step 5, 100% B for 10 min at 1 ml/min; step 6, re-equilibration of column at 65% A, 35% B at 0.4 ml/min for 15 min. AA-Ac-derivatized oligosaccharides were detected on a Waters 474 fluorescence detector at an excitation wavelength of 442 nm and an emission wavelength of 525 nm.

[2] R. Matico, S. Sweitzer, D. J. Powell, and K. Johanson, manuscript in preparation.

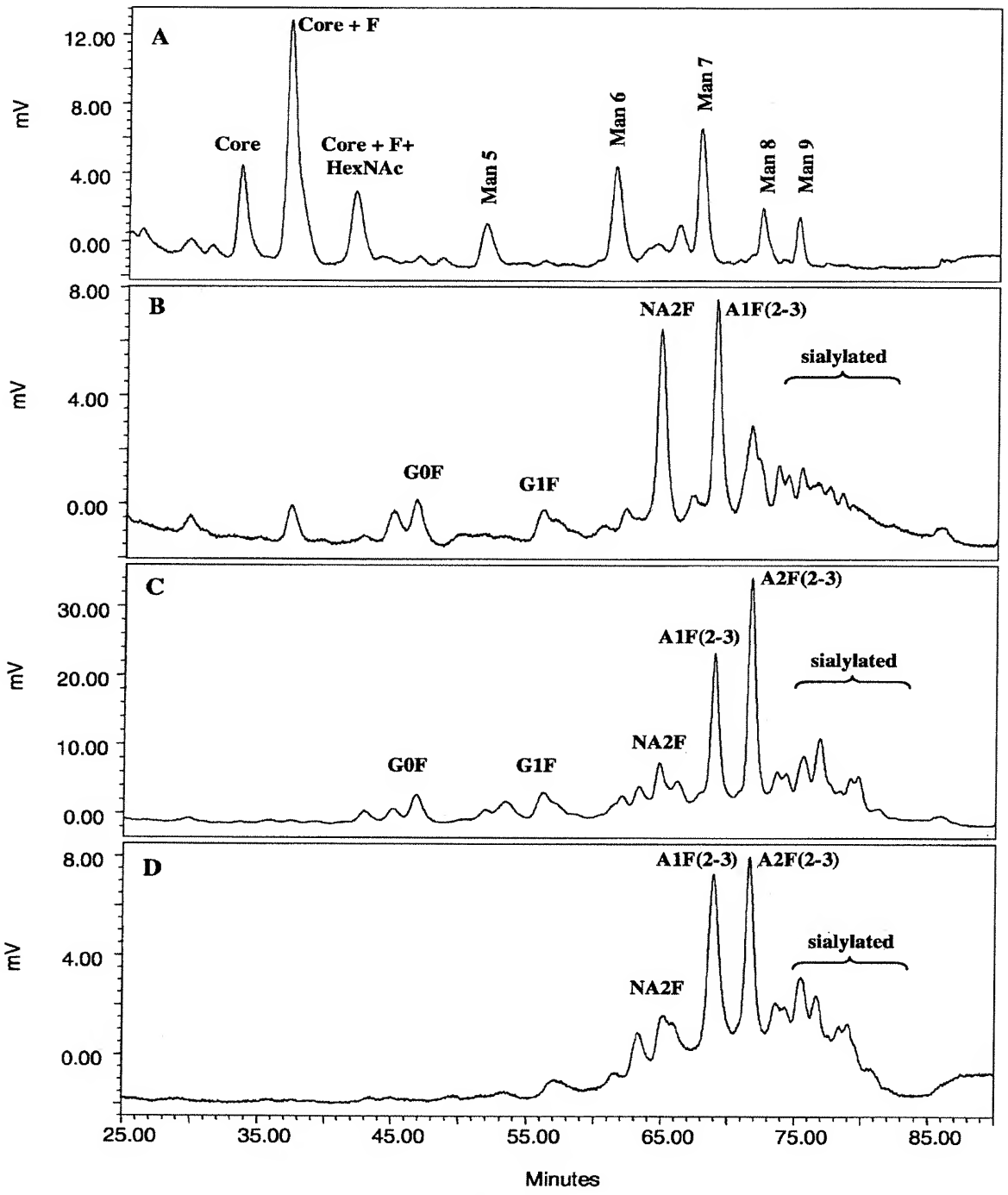
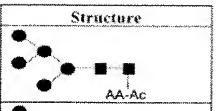
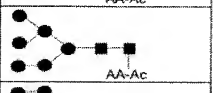
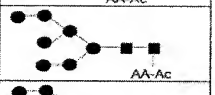
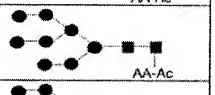
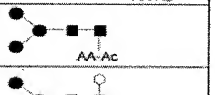
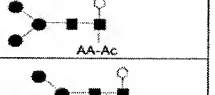
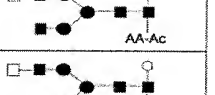
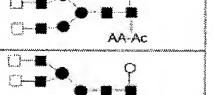
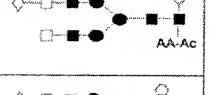
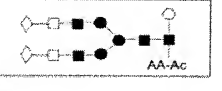


FIG. 2. **Hydrophilic interaction liquid chromatography with fluorescence detection of glycan pools released from Asp samples.** The glycans used for HILC analysis were released from wild-type Asp-2-His_6 expressed in baculovirus-infected SF9 cells (*A*), Asp-2Fc expressed in COS cells (*B*), Asp-2Fc expressed in CHO E1a cells (*C*), and purified wild-type Asp-2 expressed as an Fc fusion in CHO E1a cells with subsequent removal of the Fc moiety (*D*).

Mass Spectrometry—MALDI-TOF-MS of the derivatized glycans was carried out using a TofSpec 2E mass spectrometer (Micromass, Manchester, UK) operated in the reflectron mode. Photon irradiation from a 337-nm pulsed nitrogen laser and 20-kV accelerating voltage was used. The instrument was externally calibrated using the $[M + H]^+$ ion peaks of the peptides substance P (M_r 1347) and adrenocorticotropic hormone fragment 18–39 (M_r 2465) using an α-cyano-4-hydroxycinnamic acid matrix (10 mg/ml in acetonitrile:water (1:1)). The AA-Ac-derivatized glycans were dissolved in 10 μl of water and purified on a C18 ZipTip (Millipore Corp., Bedford, MA) for MALDI-TOF-MS analysis. Following conditioning of the ZipTip and binding of the derivatized glycans to the support according to the manufacturer's protocol, the bound glycans were washed once with 10 μl of 0.1% v/v trifluoroacetic acid and then eluted in 3 μl of 20% v/v acetonitrile containing the

TABLE I
Structure of glycans referred to in the figures and text

The following symbols have been used to identify the various sugar residues: ■, *N*-acetylglucosamine; ●, mannose; □, galactose; ○, fucose; ◊, sialic acid. Linkages between sugar residues have been removed for simplicity.

Name	[M+H]+	Structure
Man 5	1470.2	
Man 6	1632.8	
Man 7	1794.8	
Man 8	1957.9	
Man 9	2118.7	
$Man_3GlcNAc_2$ 'Core'	1146.7	
Fucosylated core	1292.7	
Core + F + HexNAc	1495.8	
G0F	1698.7	
G1F	1860.8	
NA2F	2023.0	
NA3F	2388.7	
NA4F	2753.9	
A1F	2313.9	
A2F	2605.1	

matrix 2,5-dihydroxybenzoic acid at a concentration of 10 mg/ml, directly onto the MALDI target.

RESULTS

Polyacrylamide Gel Electrophoresis and In-gel Enzymatic Digestion of Asp-2—The recombinant proteins expressed in baculovirus-infected SF9 cells, COS, and CHO E1a cells are shown in Fig. 1*A*. The baculovirus-expressed material has a COOH-terminal six-histidine tag and an apparent molecular mass of 65 kDa, whereas the mammalian expressed material has a COOH-terminal Fc tag and an apparent molecular mass of 94 kDa. The recombinant Asp2-Fc is found as an Asp2-Fc/Fc heterodimer as described (8). Under the reducing conditions employed for electrophoresis, the disulfide bond of the Fc moiety is reduced, leading to two major protein bands being visible on the gel. The 30 kDa represents the heavy chain of the Fc domain, and the band at ~94 kDa represents the recombinant Asp-2Fc protein.

The purified recombinant protein from CHO cells after removal of the Fc moiety has an apparent molecular mass of 65 kDa and is shown in Fig. 1*B*. Analysis of the carbohydrate from this material allowed for direct comparison to the baculovirus-infected material, which contained no Fc tag. The major band at about 65 kDa is a mixture of both the mature protein and mature protein in which the pro-domain has been removed. The minor protein bands migrating at ~38 and 40 kDa are recombinant protein degraded at the carboxyl terminus (present at <10%, as estimated from staining intensities). This was confirmed by MALDI-TOF-MS peptide mapping (data not shown). All recombinant Asp-2 is present as a mixture of the pro-form (amino terminus is Thr^{22}) and the mature form of the protein (amino terminus is Glu^{46}) as determined by amino-terminal sequence analysis.

After separation by electrophoresis, protein bands corresponding to recombinant Asp-2 were excised directly from the gel and oligosaccharides were released by incubating with PNGase F.[3] Following in-gel enzymatic digestion, the released glycans were extracted, dried down, and labeled with AA-Ac. This probe is highly fluorescent and is suitable for the analysis of reducing carbohydrates by normal- and reverse-phase high performance liquid chromatography techniques and by mass spectrometric methods (21). The labeled oligosaccharides were divided into three portions, two of which were digested with sialidase overnight for comparison with the original (nondesialylated) mixture.

HILC Analysis of the AA-Ac-derivatized Glycans—The mixture of AA-Ac labeled glycans released from Asp-2 and the corresponding Fc fusion protein were resolved by HILC as shown in Fig. 2. In this chromatographic method, the order of elution is related approximately to the size of the derivatized oligosaccharides; thus, smaller glycans have a shorter retention time, and others containing sialic acid residues elute later. The AA-Ac glycan profile obtained from expression of Asp-2 in the baculovirus-infected insect cells (Fig. 2*A*) differs from that of the glycoprotein expressed in CHO E1a cells (Fig. 2*D*). A comparison of the retention time of the derivatized oligosaccharides from the Asp-2 expressed in the baculovirus-infected cells with those from standard ribonuclease B glycan pool revealed that the components with an elution time of 50 min and later were the neutral high mannose structures Man 5 to Man 9 (Table I). This was confirmed by MALDI-TOF-MS (described below). The major peak at ~35 min was due to the presence of the glycan core, $Man_3GlcNAc_2$, containing a fucose attached to the *N*-acetylglucosamine residue on the reducing end of the sugar. Other lower intensity signals seen at retention times less than 45 min are due to the addition of fucose and/or *N*-acetylglucosamine to the "core" structure (see Table I).

We established that the highest proportion of glycans released from either COS or CHO E1a cells were acidic, containing one or more sialic acid residues (Fig. 2, *B–D*). These glycan structures were identified as A1F and A2F. A noticeable difference between the glycan profiles from Asp-2 and the Fc fusion protein, both expressed in CHO E1a (Fig. 2, *C* and *D*), is the appearance of the neutral glycans G1F and G0F, containing one or no galactose residues at the nonreducing end. Our previous analysis of glycans released from a number of therapeutic antibodies and other fusion proteins expressed in this cell line

[3] J. Charlwood, M. Skehel, and P. Camilleri, submitted for publication.

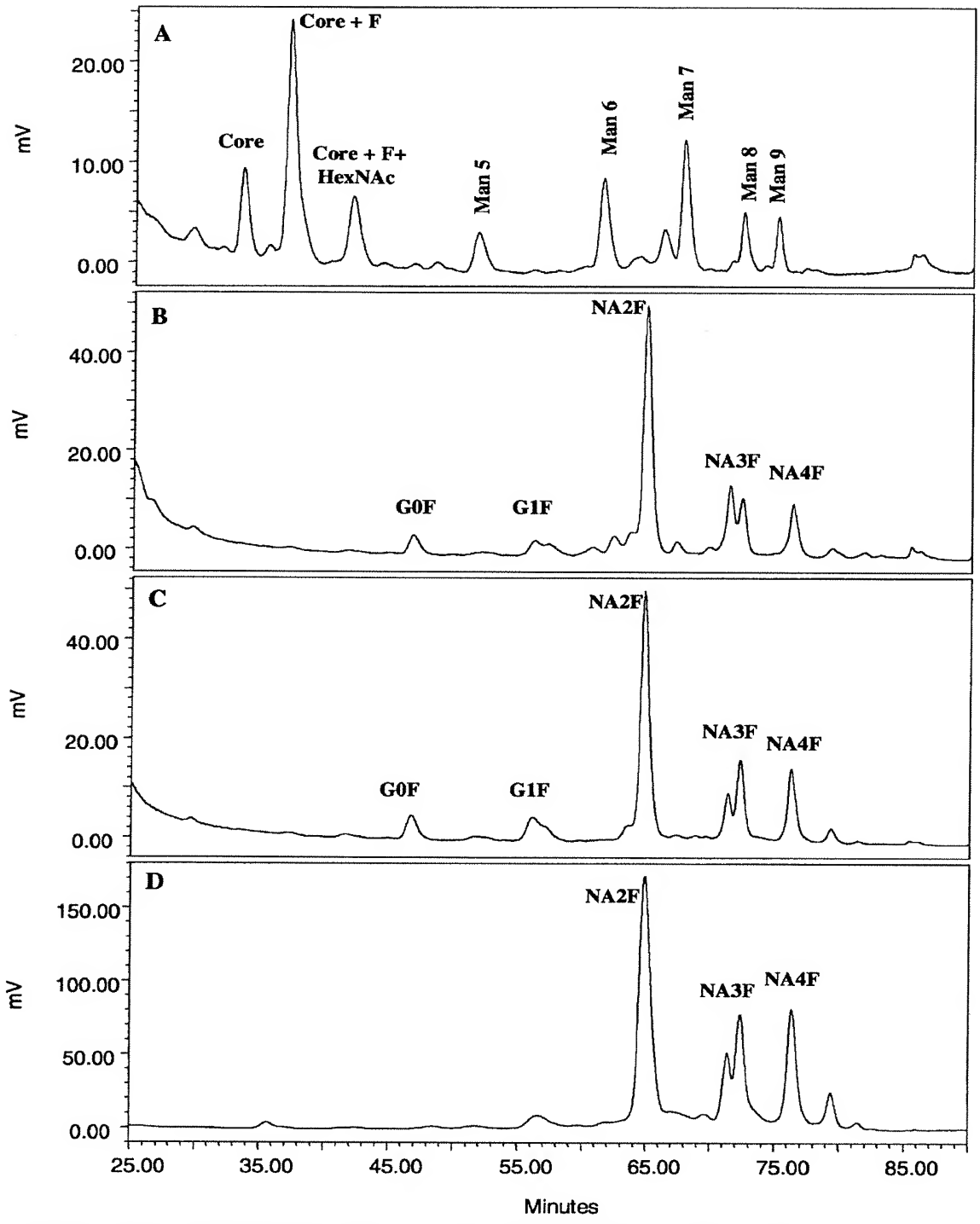


FIG. 3. **Hydrophilic interaction liquid chromatography with fluorescence detection of desialylated glycan pools released from Asp samples.** The glycans used for HILC analysis after desialylation were released from wild-type Asp-2-His_6 expressed in baculovirus-infected SF9 cells (*A*), Asp-2Fc expressed in COS cells (*B*), Asp-2Fc expressed in CHO E1a cells (*C*), and purified Asp-2 expressed as an Fc fusion in CHO E1a cells with subsequent removal of the Fc moiety (*D*).

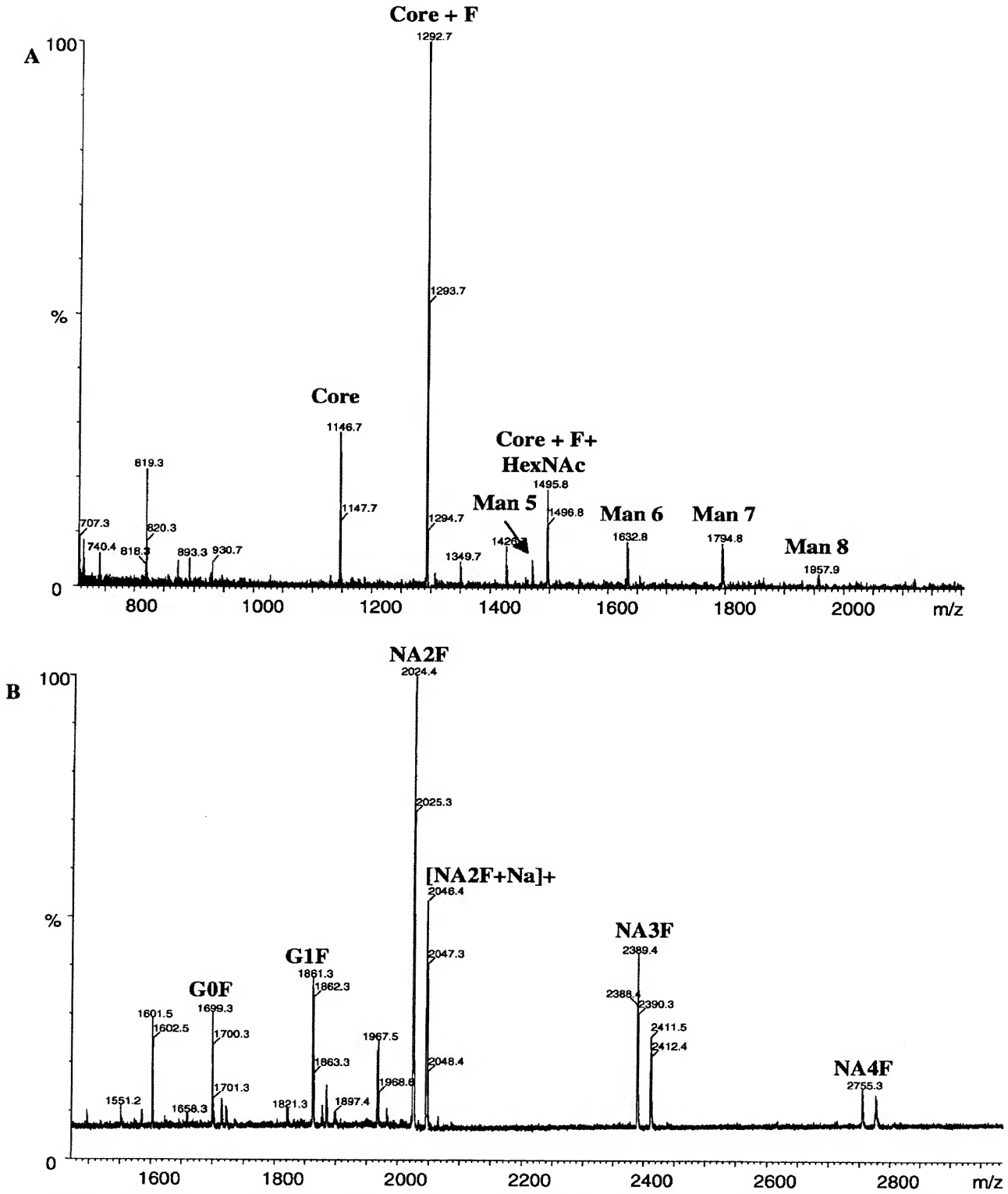
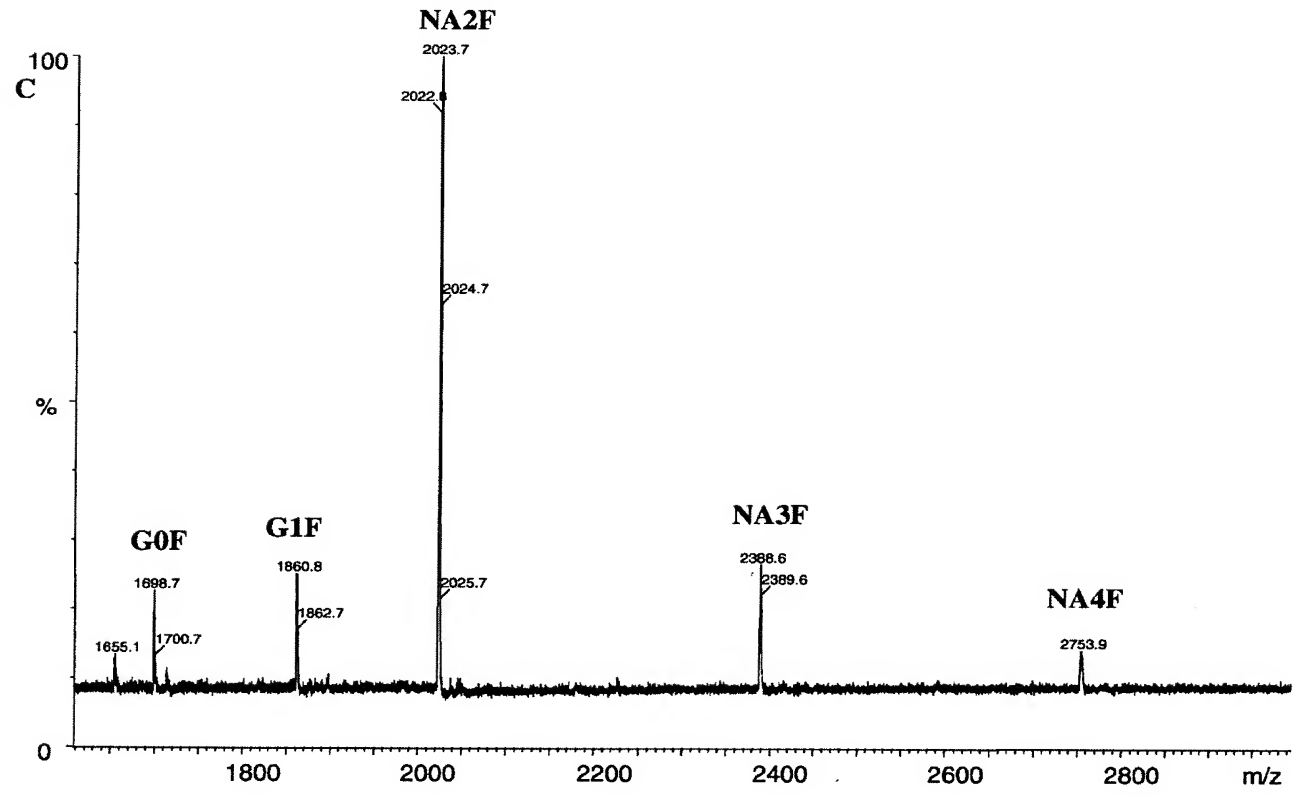


FIG. 4. **MALDI-TOF spectra of desialylated glycan pools released from Asp samples.** The glycans used for MALDI-TOF-MS analysis were released from wild-type Asp-2-His_6 expressed in baculovirus-infected SF9 cells (*A*), Asp-2Fc expressed in COS cells (*B*), Asp-2Fc expressed in CHO E1a cells (*C*), and purified wild-type Asp-2 expressed as an Fc fusion in CHO E1a cells with subsequent removal of the Fc moiety (*D*).



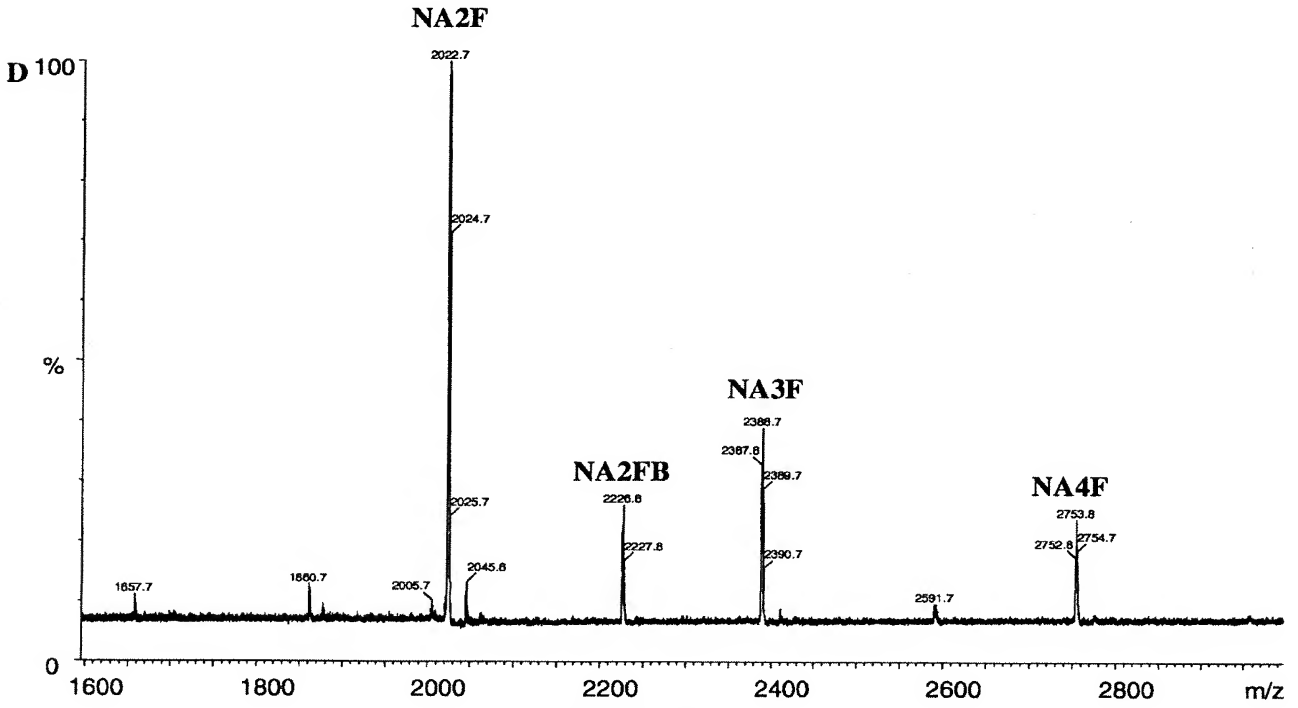


FIG. 4—*continued*

TABLE II
Activity of Asp-2 mutants

Asp-2Fc mutants which lack specific glycosylation sites were analyzed for proteolytic activity against an APP Swedish variant peptide (ISEVNLDAEFRHDK(dnp)G). Proteolytic activity is the mean of duplicate incubations.

Enzyme	Activity
	nmol/min/mg
N172Q, N223Q	30
N223Q, N354Q	23
N153Q, N172Q	23

indicates these two biantennary oligosaccharides are attached to an asparagine residue on the Fc moiety.[3]

On digestion with sialidase, the glycan mixture released from Asp-2 expressed in baculovirus-infected insect cells was identical to that before treatment (compare Figs. 2*A* and 3*A*). This confirmed that only neutral glycans are produced in this expression system. In contrast, the chromatographic profile of the derivatized glycans from Asp-2 (or the Fc fusion protein) synthesized in the CHO E1a and COS cell lines was considerably simplified after sialidase treatment (compare chromatograms in Figs. 2 and 3). Based upon the retention times of the peaks compared with those of AA-Ac-derivatized standards, these glycans were provisionally identified as NA2F, NA3F, and NA4F. The schematic structures of these oligosaccharides are shown in Table I.

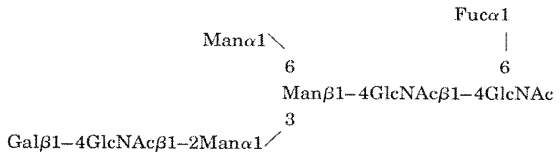
MALDI-TOF Mass Spectrometric Analysis of AA-Ac-derivatized Glycans—Molecular weight analysis of the individual components in the mixtures of the AA-Ac-derivatized neutral glycans by MALDI-TOF mass spectrometry provided detailed structural information on the corresponding heterogeneity and the identity of the individual glycans.

The mass spectrometric analysis of the neutral glycan mixtures released from Asp-2 and its Fc fusion protein is shown in Fig. 4. The majority of AA-Ac glycans were observed as the proton adducts, $[M + H]^+$, although in the case of glycans from the COS cell expression a sodium adduct was also identified (Fig. 4*C*). The profile obtained for the oligosaccharide mixture from the protein expression in baculovirus-infected insect cells confirmed the presence of most of the glycans identified by their chromatographic behavior. Thus, the major glycans from this cell line were confirmed as oligomannose-type oligosaccharides.

The profiles of the desialylated glycan mixtures released from the Fc fusion protein synthesized in COS and CHO E1a, are shown in Fig. 4 (*B* and *C*, respectively). From the chromatographic and MALDI data, it is clear that the distribution of components in the two mixtures are very similar and contain bi-, tri-, and tetra-antennary complex-type glycans, which can be sialylated at the galactose residues on the nonreducing ends of these molecules. The heterogeneity of the major glycans released from Asp-2 expressed in CHO E1a is also similar to that of the Fc fusion protein from the same cell line (Fig. 4*D*). The MALDI-TOF-MS data are summarized in Table I.

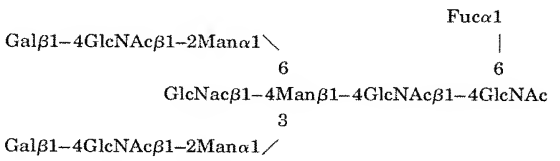
Three minor signals with m/z values of 1657.7, 2226.8, and 2591.7 were observed in the MALDI-TOF-MS of the glycan profile released from Asp-2 (Fig. 3*D*). These signals were largely absent in the Fc fusion protein. The low level increase in heterogeneity is most likely due to small differences in the fermentation conditions used to prepare a larger batch of Asp-2 Fc before proteolytic cleavage of the Fc moiety.

Loss of an *N*-acetylglucosamine residue (203 mass units) from G1F (m/z 1860.7) gives m/z 1657.7. This probably has the structure shown below (Structure I), an uncommon glycan that has also recently been identified as one of the glycan components released from gelatinase B (22).



STRUCTURE I

The component with an $[M + H]^+$ value of 2225.8 is 203 mass units higher than that of the neutral biantennary glycan NA2F. This is most probably related to the presence of low levels of a bisected glycan (Structure II), although the exact position of the *N*-acetylglucosamine position cannot be determined from these measurements.



STRUCTURE II

The very low level glycan component at m/z 2591.7 is about 162 mass units (galactose residue) lower in molecular weight than NA4F. The presence of this oligosaccharide may be due either to the incomplete processing of the tetra-antennary complex-type glycan or to the formation of triantennary complex-type glycan with an *N*-acetylglucosamine residue attached to the central mannose residue in the mannose core.

Activity Analysis of Glycosylated and Unglycosylated Asp-2—Site-directed mutagenesis was used to alter four asparagine residues (amino acids 153, 172, 223, and 354) to glutamines, within the consensus glycosylation sites of Asp-2Fc. The mutants were expressed and purified from COS cells, and the activity was compared with the wild-type Asp-2Fc (Table II). Three double mutants, which eliminated two of the four glycosylation sites, showed reduced proteolytic activity (between 30% and 40% of wild-type).

DISCUSSION

The glycosylation state of Asp-2 plays a critical role in maintaining its proteolytic activity. In our initial experiments, tunicamycin treatment of a stable CHO cell line expressing Asp-2Fc was used to obtain unglycosylated Asp-2Fc. This protein exhibited ~40% of the activity of the Asp-2Fc isolated from untreated cells (data not shown). This protein is unaltered in its amino acid sequence but has greatly reduced carbohydrate content (data not shown). These results suggest that glycosylation may play an important role in the proteolytic activity of Asp-2. Although glycosylation could not be detected from the tunicamycin-treated Asp-2Fc, there was a possibility that this sample was not homogeneous in its glycosylation state. Therefore, to address this question more rigorously, we undertook to mutate the glycosylation sites on Asp-2Fc.

Mutating two of the four consensus glycosylation sites of Asp-2 had a significant effect on proteolytic activity. The effect of glycosylation on enzyme activity could be indirect, perhaps by increasing the solubility of the Asp-2 protein. However, it is also possible that the glycosylation could have a direct effect on substrate binding. According to the crystal structure of Asp-2, asparagine 172 is within 10 Å of the unprime side of the binding pocket and asparagine 223 is within 13 Å (23). A complex carbohydrate, such as A2F, has dimensions in excess

of 30 Å, allowing for the possibility of a direct interaction with peptide substrates and a likely interaction with the larger surface of the natural substrate, APP.

A number of other observations support the effect of glycosylation on activity. The baculovirus-expressed Asp2, which has high mannose glycosylation, showed ~50% of the activity of the mammalian expressed material, which contains complex carbohydrates (data not shown). This may suggest that it is not only important to have carbohydrate at these sites, but that specific sugar moieties may be important for a direct interaction with substrate. In addition, the refolded *E. coli* expressed Asp-2, which is not glycosylated (24), was assayed at a 10-fold higher concentration than our experiments, suggesting that its activity is significantly lower than the mammalian expressed protein. The *E. coli* expressed material contains asparagine residues at all four consensus glycosylation sites, again supporting the hypothesis that the lack of glycosylation and not the alteration of the asparagine residues is leading to the decrease in activity of Asp-2.

Each of the three double mutants analyzed shows a significantly reduced activity, suggesting that multiple glycosylation sites play a role in maintaining optimal activity of Asp-2. Although this analysis does not address the individual contributions of each of the glycosylation sites, it does show that more than one carbohydrate effects activity and implies that some of the glycosylation effect is indirect. Initial attempts at mutating all four of the consensus glycosylation sites in Asp-2 were problematic due to low levels of protein expression. This could be due to a retardation in the rate of secretion of this protein, or an increase in the lability of the protein once it is secreted.

The chromatographic behavior of the intact and desialylated mixture of glycans released from Asp-2 expressed in mammalian cell reveals that the majority of these glycans are of the complex type, containing one or more sialic acid residues. Retention time measurements of the neutral glycans obtained after desialylation gave a preliminary indication of the identity of these oligosaccharides as bi-, tri-, and tetra-antennary parents. Molecular weight measurements by MALDI-TOF mass spectrometry determined the molecular weight of the components in these mixtures, providing further evidence for their identity.

The use of AA-Ac in preference to other fluorescent labels, such as 2-aminoacridone, has provided an increase of approximately an order of magnitude in sensitivity to MALDI-TOF-MS measurements (21). Due to this higher sensitivity, it has been possible to reveal the presence of other lower level carbohydrates attached to the *N*-glycosylation sites of Asp-2.

In contrast to the expression of Asp-2 in mammalian cell lines, synthesis in baculovirus-infected insect cells produced only neutral oligosaccharides containing three or more mannose residues. This result is in agreement with the preponderance of evidence in the literature that *N*-glycosylation in insect cells is limited to almost exclusively oligomannose-type glycans ($Man_{3-9}GlcNAc_2$) (25).

Limited information on the glycosylation of Asp-2 has been published to date. One study reported that enzymatic digestion of this glycoprotein with endoglycosidase H did not lead to deglycosylation (12). This is not surprising as endoglycosidase H will only hydrolyze high mannose carbohydrates, whereas the glycans identified in this study are of the complex type when expression of the protein is carried out in a mammalian cell line. In another study it was shown that all the four *N*-glycosylation sites on Asp-2 are occupied by a heterogeneous mixture of carbohydrate (8). These authors found that four oligosaccharides were linked to Asn^{172}. The molecular weights of these glycans differed from one another by 162 and 366 mass units, which indicated the loss of a hexose and of a disaccharide made up of hexose and *N*-acetylhexosamine, respectively. These differences are in agreement with the type of bi-, tri-, and tetra-antennary complex structures we identified in our investigation, as shown in Table I. For example, the difference in molecular weight between a bi- and a tri-antennary glycan is 366, as is the case for tri- and tetra-antennary glycans. The presence and extent of sialylation of these complex-type glycans could not be estimated from this recent study (8).

Knowledge of the type of carbohydrate covalently attached to asparagine residues in Asp-2 has also been useful in the design of a purification protocol. It has allowed a rational choice of the correct lectin column for the purification of recombinant Asp-2, and has helped to establish a suitable protocol for the crystallization of this protease.

It is now widely accepted that the biological functions of carbohydrate covalently attached to proteins are known to be diverse (9). They can ensure the correct folding of a protein by stabilizing folded domains, they can enhance the solubility of a protein by providing polar groups on its surface, they can prevent or diminish aggregation of the glycoprotein, they can protect the protein from protease degradation, and can modulate intracellular routing and intercellular recognition. A key question is whether the large size of the glycan moieties in Asp-2 compared with the protein domain has any influence on its β-secretase properties either due to direct interaction with APP or indirectly by ensuring its correct folding in the endoplasmic reticulum.

In conclusion, we have shown that the β-secretase activity of Asp-2 is dependent on the extent of *N*-glycosylation. This result was derived by expressing Asp-2 in the presence of tunicamycin and was further confirmed when the protease activity of mutants were assessed using a peptide substrate. It is well known that a number of glycoproteins need one or more of their *N*-linked oligosaccharides during folding (12). The fact that protease activity was affected by the occupancy of glycosylation sites, close and further away from the site of action, appears to indicate that all four glycosylation sites in Asp-2 must be occupied by oligosaccharide and act in a cooperative manner to make sure that this glycoprotein has the correct folded conformation (12, 20). We will be carrying out future studies to find out whether one or more of the glycosylation sites plays a crucial role in the correct folding of Asp-2. As two of the *N*-glycosylation sites, namely Asn^{223} and Asn^{354}, are spatially close to the disulfide bridges Cys^{216}-Cys^{420} and Cys^{278}-Cys^{443}, respectively, the presence of the correct type of oligosaccharide may also play an active role in the formation of disulfide bridges and hence can have an effect on the tertiary structure of the glycoprotein. To fully appreciate the impact of glycosylation on protease activity, it will also be important to ascertain the glycosylation states of the mammalian-expressed full-length Asp-2, as well as native Asp-2. The technology presented here makes analysis of the native protein possible because small amounts of protein can be easily analyzed.

Glycosylation of Asp-2 seems to be essential in the maintenance of an active conformation of this protein, ensuring optimum interaction and β-secretase reactivity with APP. Clearly establishing the precise role of glycosylation in the protease activity of Asp-2 will be important in the design of specific inhibitors that may more effectively interfere with the function of Asp-2. This knowledge will be useful in structure-activity relationships, which will be important in optimizing the therapeutic value of drug treatments for Alzheimer's disease.

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